AD_____

Award Number: W81XWH-04-1-0407

TITLE: Examining the Role of Msh2 and Mre11 in Telomere Rescue

PRINCIPAL INVESTIGATOR: Damon Meyer

CONTRACTING ORGANIZATION: Beckman Research Institute
Duarte, California 91010-3000

REPORT DATE: April 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2005	Annual Summary (15 Mar 04 - 14 Mar 05)

4. TITLE AND SUBTITLE
Examining the Role of Msh2 and Mre11 in Telomere Rescue

5. FUNDING NUMBERS
W81XWH-04-1-0407

6. AUTHOR(S)
Damon Meyer

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beckman Research Institute
Duarte, California 91010-3000

E-Mail: dmeyer@coh.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Interestingly, about 15% of human cancers have an inactive telomerase gene, leading to the discovery of telomerase-independent mechanisms for regaining telomeric sequences by homologous recombination referred to as alternative lengthening of telomeres (ALT). In *S. cerevisiae*, there are two ALT pathways controlled by Rad51 and Mre11/Rad50/Xrs2 (MRX). The ALT pathway used in humans generates telomeres that resemble the MRX-dependent survivors found in yeast. Recent evidence suggests that the mismatch repair pathway, which is thought to block recombination between mismatched telomeric sequences, limits ALT, perhaps by opposing MRX-dependent ALT.

I propose to examine the role of Msh2, the central mismatch repair protein, in restricting ALT in *S. cerevisiae*. Particular attention will be paid to whether Rad51 or MRX pathway recombinants predominate in these strains, suggesting that mismatch repair selectively restricts one pathway or the other. In addition, the role of Mre11 in ALT will be studied by determining the effect of mutations in specific functional domains of Mre11 in telomerase-independent telomere rescue. This may help determine the relationship between MRX-dependent ALT and specific homologous recombination mechanisms.

14. SUBJECT TERMS
Genetics, Molecular Biology, DNA Repair, Homologus Recombination, Genome Instability, Telomere Rescue, Mismatch Repair, Mrell/Rad50/Xrs2

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction: Study of the genetic requirements for recombination-dependent telomere rescue by ALT (alternative lengthening of telomeres) is critical for understanding the events that lead to the development of telomerase-negative breast cancer, which accounts for ~15% of all breast cancer (1). Both humans and *S. cerevisiae* have been shown to undergo ALT, which is in turn dependent on a variety of proteins that are conserved between the two organisms (1) (2) (3). Two of these proteins; Mre11 and Msh2, are thought to be involved in promoting recombination of telomeres, and in restricting telomere recombination to homologous sequences respectively (3) (4). This study will help define the role of Msh2 in restricting ALT, and the role of Mre11 in propagating it.

Body:

Task 1: Creation of the mutant strains needed for the study.

a. I have created two mutant strains of *EST2* by disrupting the coding sequence with one of two selectable prototrophic markers, *URA3* or *LEU2*, resulting in *est2::URA3* and *est2::ura3::LEU2.* These allow for the use of two different selectable markers when creating mutant strains with *MSH2* and/or *MRE11.* In addition, the *est2 msh2* double mutant and the *est2 msh2 mre11, est2 msh2 rad51* and *est2 mre11 rad51* triple mutants have been created. Creation of strains containing *msh2* mutants was problematic since these mutant strains are hypermutagenic. To get around this only fresh single colony cells were used to make the appropriate crosses.

Task 2: Examine the role of Msh2 in restricting ALT in *S.cerevisiae.*

a. Currently I have done serial liquid growth and streaking on to YPD plates for wild-type, *est2* and *est2 msh2* cells. In addition, cell viability assays were done each day for liquid cultures of wild-type, *est2* and *est2 msh2* cells. The results are shown below (Fig. 1 and 2). It appears from the data that *est2 msh2* double mutants do not senesce to the level observed in the *est2* mutants. This is most evident when examining cell viability in Fig. 2, which requires single cells to form a colony on YPD plates, unlike the serial liquid growth assay that just counts cell bodies. Cell viability in *est2 msh2* double mutants drops to around 25-35% and remains low until 100 generations, unlike *est2* mutants that show a more dramatic decrease in cell viability and a more rapid recovery (60-70 generations). Furthermore the growth of *est2 msh2* in liquid culture is slightly more robust than *est2* during the time of senescence from 40-60 generations. However, both *est2* and *est2 msh2* recover from senescence by around 80 generations, with *est2 msh2* mutants having a slightly lower cell density than *est2.*

Figure 1: *est2 est2 msh2* and WT growth in liquid culture

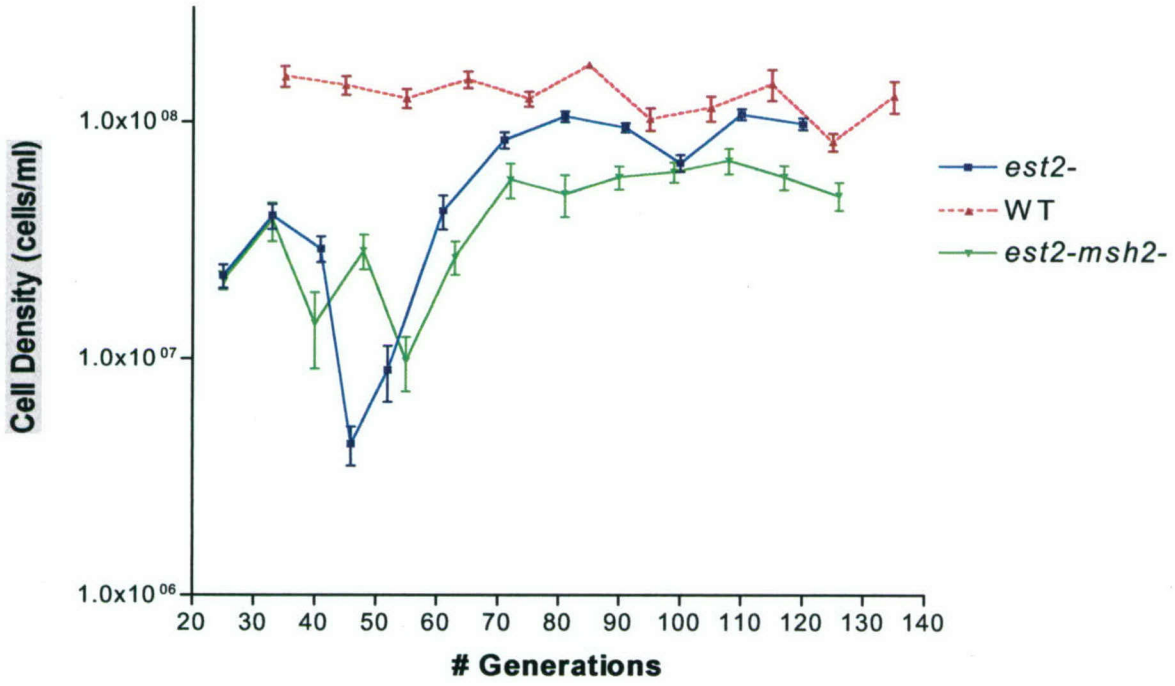
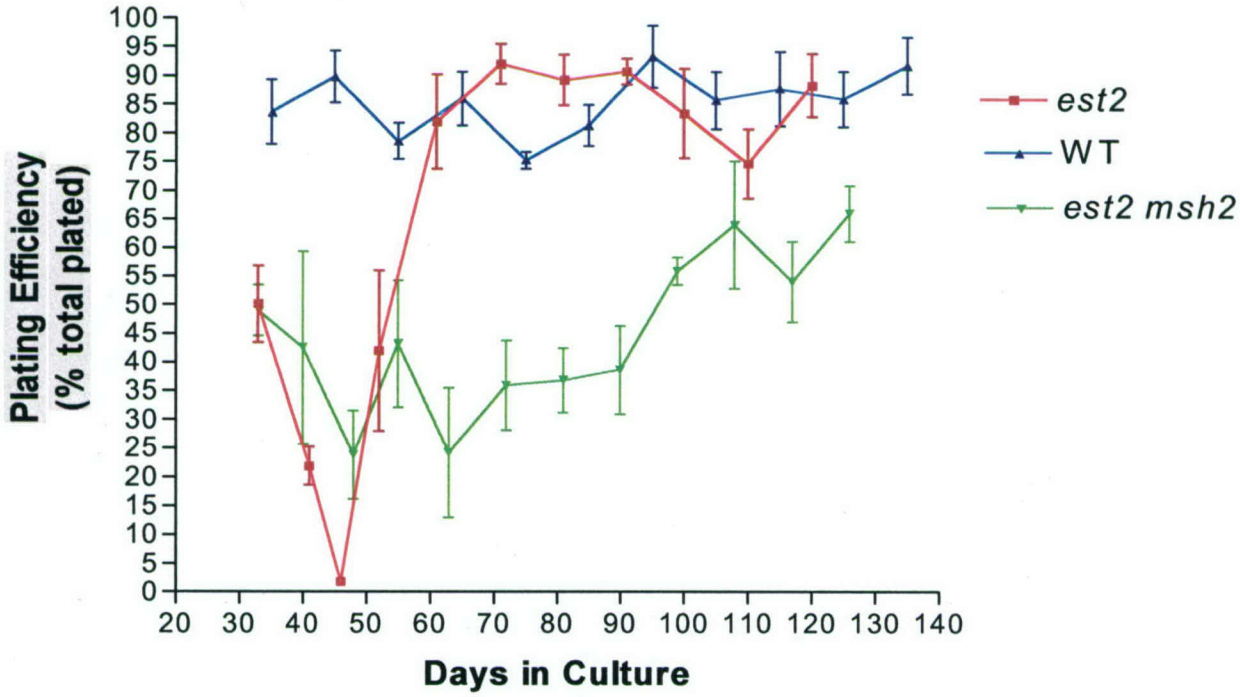


Figure 2: Cell Viability



b. Determining the advent of survivors in *est2* and *est2 msh2* mutants during continuous liquid culture is in progress and should be finished in 1-2 months. In addition, survivors of *est2* and *est2 msh2* have been examined to determine the ratio of Type I/ Type II survivors and if loss of *MSH2* changes that ratio. The results are summarized below (Table 1: Appendices) and represent data derived from survivors in liquid culture and from plates. In *est2* survivors the relative ratio of Type I/ Type II survivors is 23/50 = .46. However, *est2* msh2 survivors have a relative ratio of Type I/ Type II survivors of 33/1 = 33 a significant shift from that observed in *est2* survivors. These data suggest that Msh2 is either involved in restricting Type I survivor formation, or in promoting the formation of Type II survivors. This difference can only be addressed upon final examination of *est2 msh2 rad51* and *est2 msh2 mre11* triple mutants.

c. I have just begun examining which ALT pathway Msh2 is involved in by first comparing the generation of survivors in *est2 msh2 rad51* triple mutants to *est2 rad51* and *est2 msh2* double mutants. Preliminary results show no noticeable difference in serial liquid or plate growth between *est2 msh2 rad51* and *est2 rad51*. However, since *est2 msh2* mutants do show an improvement in growth relative to *est2 rad51* and *est2 msh2 rad51* these findings suggest Msh2 has no role in promoting Type II survivor formation but rather restricts the formation of Type I survivors. Further work must be done to make any definitive conclusions.

Task 3: Test the involvement of specific Mre11 domains in ALT.

a.-c. Currently I have not yet started work on this phase of the research since I have focused on determining the involvement of Msh2 in ALT and its relationship, if any, to Mre11.

Recommended Changes/ Future Work:

In an effort to better understand the events that occur during senescence and the generation of survivors in *est2* mutants, three additional experiments are being done.

I. *Measuring the Frequency of Telomere Recombination.*

Currently there is no quantitative assay that examines spontaneous telomere recombination. Therefore, I have begun to construct an assay that will allow for the measurement of telomere recombination frequency. Determining the frequency of telomere recombination and its genetic control is critical in understanding ALT-dependent cancer progression. I plan on examining the role of Msh2 and Mre11 in telomere recombination during the progression toward senescence and subsequent survivor formation in *est2* mutants. Being able to determine the frequency of telomere recombination will allow for a more sensitive investigation of inhibitors and enhancers of this process. Ultimately, this may allow for the identification of the best potential targets for the inhibition of ALT.

The assay is shown below (Fig. 3, Appendices) and consists of a selectable marker, *TRP1*, at the end of the right arm of chromosome V. Just interior to *TRP1* is the acceptor sequence, *his3Δ3'*, that is missing ~160bp at its 3' end. The *HIS3* gene is located on the arm of chromosome XV. The donor sequence, *his3Δ5'*, consisting of the 3' end of the *HIS3* gene. The amount of homology between the acceptor, *his3Δ3'*, and donor, *his3Δ5'*, is 500bp. This is sufficient enough homology to allow for efficient recombination to occur between the two sequences. In addition, the native *HIS3* gene will be deleted to prevent its use as a donor in the assay. Telomere recombination will be measured by the appearance of *HIS3* colony formation when cells are grown on medium lacking histidine. Currently I have completed the first of three cloning steps needed for the creation of the telomere construct.

II. *Measuring Genome Instability by Chromosome Loss, Mutations and Gross Chromosomal Rearrangements.*

As *est2* mutants progress toward senescence it is thought that telomeres progressively shorten leading to a loss of "capping" and thus DNA end protection. As a result unfavorable genomic rearrangements occur leading to replicative senescence. This genomic instability also occurs in human somatic cells, which have continued to divide beyond the normal control of cell growth. These cells are precursors in cancer development. Although most die, a few may potentially acquire genetic changes that allow them to progress toward a cancerous state, which includes ALT-dependent cancer. Therefore, it is of interest to study chromosome and telomere specific genomic instability, by examining chromosome loss and gross chromosomal rearrangements (GCR) respectively.

The chromosome loss assay is shown below (Fig. 4, Appendices), and makes use of two selectable markers on opposite ends of the centromere on chromosome V. Loss of both selectable markers will be counted as a chromosome loss event while loss of one marker will be counted as a gene conversion/BIR event. Analysis of chromosome loss will be done in a diploid since this event is lethal in a haploid. Currently I am in the last step of completing the chromosome loss strain at which time I can test the effect of *est2* on chromosome loss.

In addition to chromosome loss I am also examining the role of *est2* in GCR and mutations at the telomere. The GCR/mutation assay shown below (Fig. 5, Appendices) uses two markers, *CAN1* and *hxt13::URA3*, located 21kb and 13kb away from the telomere respectively. Cells with the appropriate genotype are taken from the dissection plate and plated to YPD and medium containing canavinine. Cells on YPD plates that form colonies will be counted to determine the number of generations the original colony had gone through, and then a fresh colony from the YPD plate will be used to plate to YPD and medium containing canavinine. This process will be repeated five times, or from 0-125 generations. Mutations are measured as a *CAN1* forward mutation, which allows for cells to grow on medium lacking arginine and containing canavinine, a poisonous arginine analog able to be transported into the cell by Can1. Mutations in *CAN1* prevent canavinine from entering the cell leading to colony formation. GCR is measured by taking *can1* mutants and replica plating them to medium lacking uracil. Cells that are *can1 ura3* have lost ~21kb of telomeric DNA and are scored as a GCR

event. Currently I have examined wild-type and *est2* cells for both mutations and GCR the results of which I have summarized below (Fig. 6 & 7). The results indicate that, as *est2* mutants progress toward senescence there is a large increase in mutation rate, 37-fold, and an even greater increase in GCR, 180-fold. Furthermore, it appears that as *est2* cells begin to generate survivors both mutagenesis and GCR decrease back to wild-type levels by about 125 generations. I also looked at survivors of >150 generations and they also seem to show levels of mutations and GCR that have been restored to wild-type levels. Interestingly, a few survivors examined took longer to show decreases in GCR and mutations than the average population, indicating that the survivor population is highly dynamic with respect to telomere stability, which depends on favorable telomere recombination events. Currently, we are attempting to discover the mechanism behind the increase in mutation and GCR rate by attempting to correlate an increase in ssDNA at telomeres with the observed increases in mutations and GCR.

Key Research Accomplishments:

- Generated a *est2::ura3::LEU2* allele that uses a new marker to follow *est2* mutants.
- Recapitulated results by Rizki & Lundblad (2001) showing *est2 msh2* cells grow better than *est2* during the time of senescence in liquid culture .
- Showed cell viability in *est2 msh2* double mutants is low but stable at around 25-35%, has a delayed recovery around 100-110 generations.
- Cell viability in *est2* mutants follow a similar pattern of cell growth, senescence and recovery observerd in serial liquid growth.
- The relative ratio of Type II/I survivors in *est2* mutants is 23/50 = .46.
- The relative ratio of Type II/I survivors in *est2 msh2* double mutants is 33/1 = 33.
- Completed the first of three cloning steps needed for the creation of the telomere construct.
- Working on the last step in the creation of an *est2* chromosome loss strain.
- Showed a 37-fold increase in the mutation rate of a telomere proximal gene in *est2* mutants during the time of senescence. Furthermore, the mutation rate of a telomere proximal gene decreased to wild-type levels during survivor formation.
- Showed a 180-fold increase in the GCR rate in *est2* mutants. This increase occurred during senescence and progressively decreased during the advent of survivor formation. Determined that there is variability in the number of generations *est2* cells need to stabilize their telomeres (125-200 generations).

Reportable Outcomes: None currently.

Conclusions:

The results from the completed research begin to shed light on the role of Msh2 in restricting ALT and the stability of telomeres during the progression toward ALT survivors. The role of Msh2 appears to be more complex then a general restriction of telomere recombination as determined previously (4). In an effort to better understand the role of Msh2 in ALT we examined *est2* and *est2 msh2* mutants in a serial liquid growth assay, cell viability for each day of growth and the relative ratio of Type I/ Type

II survivors. Our results from the serial liquid growth and cell viability assay indicate *est2 msh2* double mutants never undergo replicative senescence, but maintain a stable growth state until survivor formation 40-50 generations after normal survivor formation is seen in *est2* mutants. Furthermore, examination of the relative ratio of Type I/Type II survivors in *est2* and *est2 msh2* mutants showed a dramatic shift from favoring Type II survivors 23/50 to favoring Type I survivors 33/1 respectively. These results suggest Msh2 is either restricting ALT in a way that favors Type II survivors, or that Msh2 is promoting Type II survivors. Only upon examination of the triple mutants *est2 msh2 rad51* and *est2 msh2 mre11* can a determination be made between the two possibilities. However, early results with the *est2 msh2 rad51* and *est2 rad51* mutants show no growth difference between the two supporting a role for Msh2 in restricting ALT in a way that favors Type I survivors. If Msh2 was needed to promote Type II survivors then the *est2 msh2 rad51* triple mutant might be synthetically lethal or morbid.

Understanding telomere stability in *est2* mutants is critical in attempting to determine the events leading to cancer progression. In order to analyze telomere stability a GCR assay was performed on *est2* mutants as they progressed toward survivor formation. Our results show a significant increase in both *CAN1* mutation and GCR in *est2* during the time of senescence and a subsequent decrease as survivors are generated. Interestingly, while *CAN1* mutation rate decreases to wild-type levels within ~25 generations, the GCR rate decreases progressively suggesting the mutation rate is due to senescence, but that GCR is a result of continued telomere recombination needed for survivor formation. However, *est2* survivors eventually have wild-type mutation and GCR rates, indicating that a new, stable population has arisen. These results shed light on the progression of cancer development in the absence of telomerase. In human somatic cells that lack telomerase, telomere instability increases leading to GCR and mutations. In this context cells have the possibility of acquiring genetic changes that ultimately may lead to cancer. This also has an additional implication with respect to the evolution of gene location along linear chromosomes. If proximity to the telomere results in senescence-related mutations and GCR then the telomere proximal genes may be susceptible to these events. We are investigating whether this has had any impact on the distribution of esssential genes in the genome.

"So What"

These results are helping us to develop an understanding of how senescent somatic cells may progress toward cancer. This will help to identify molecular targets for drugs that attenuate the growth of these unique ALT cancer cells that would be immune to telomerase inhibitors.

References:

1. **Neumann, A.A., Reddel, R.R.** 2002. Telomere maintenance and cancer look, no telomerase. Nature Reviews **2**:879-884.
2. **Teng, S., and Zakian, V.** 1999. Telomere-Telomere Recombination Is an Efficient Bypass Pathway for Telomere Maintenance in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **19**:8083-8093.
3. **Le, S., Moore, J.K., Haber, J.E., and Greider, C.** 1999. RAD50 and

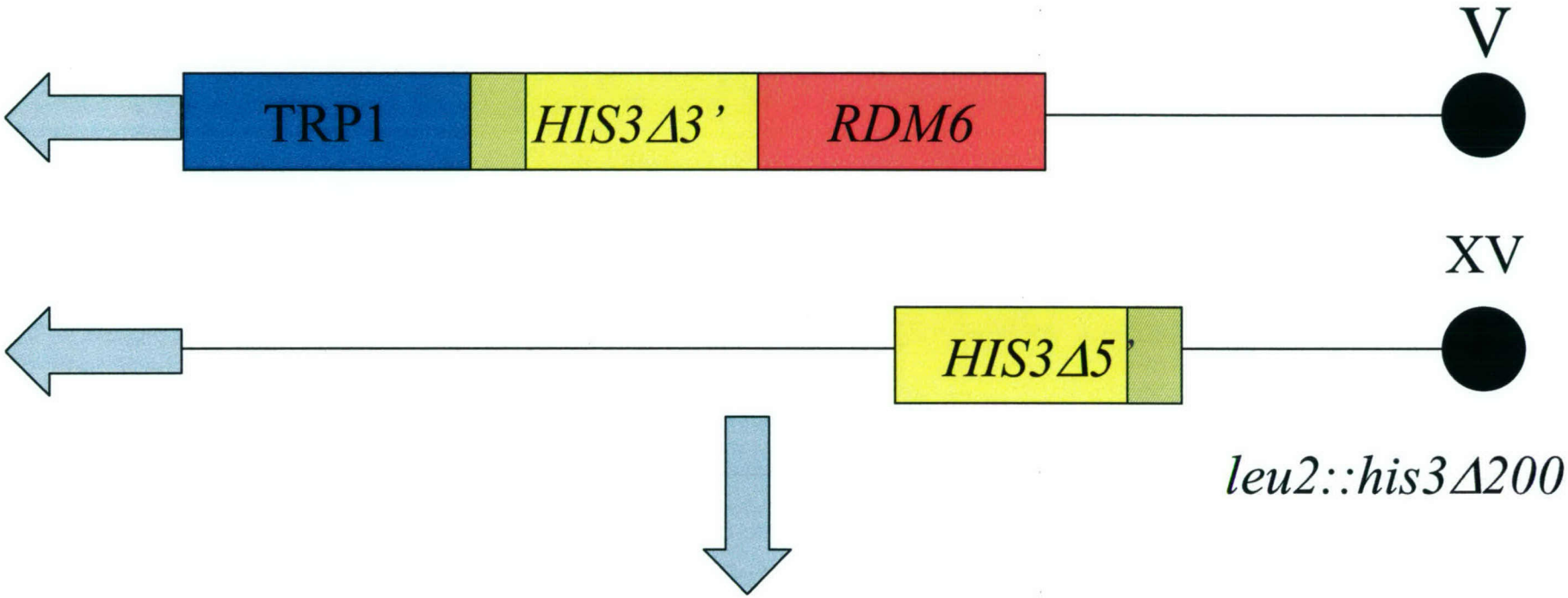
RAD51 Define Two Pathways That Collaborate to Maintain Telomeres in the Absence of Telomerase. Genetics **152**:143-152.

4. **Rizki, A., Lundblad, V.** 2001. Defects in mismatch repair promote telomerase-independent proliferation. Nature **411**:713-716.

Table 1 : Relative ratio of Type I to Type II survivors

	Type I	Type II	Type I/II
est2	23/73 = 31.5%	50/73 = 68.5%	23/50 = .46
est2 msh2	33/34 = 97%	1/34 = 3%	33/1 = 33

Fig. 3 Telomere Recombination Assay



Look for *HIS*+ recombinants

Fig. 4 Chromosome Loss Assay

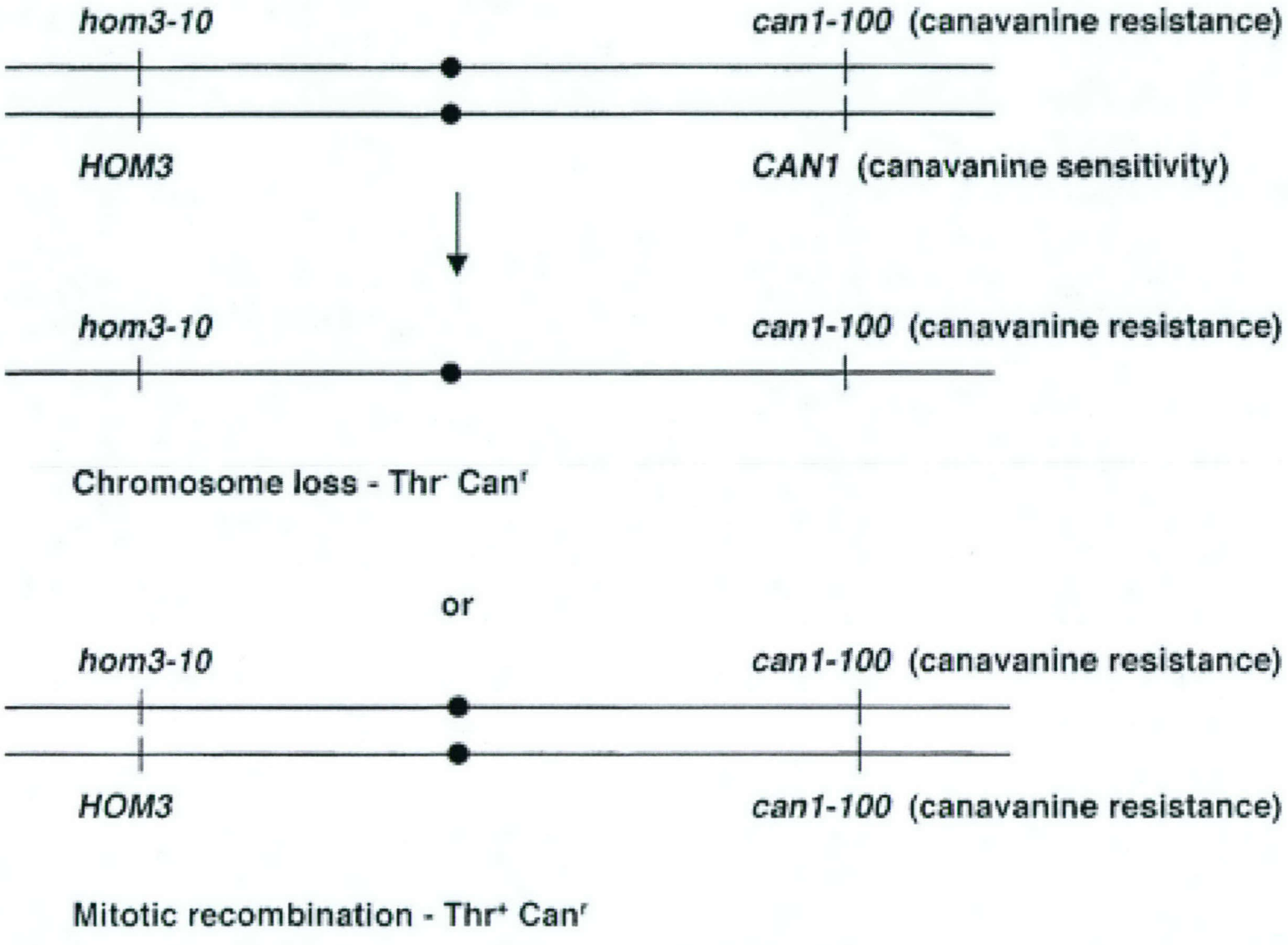
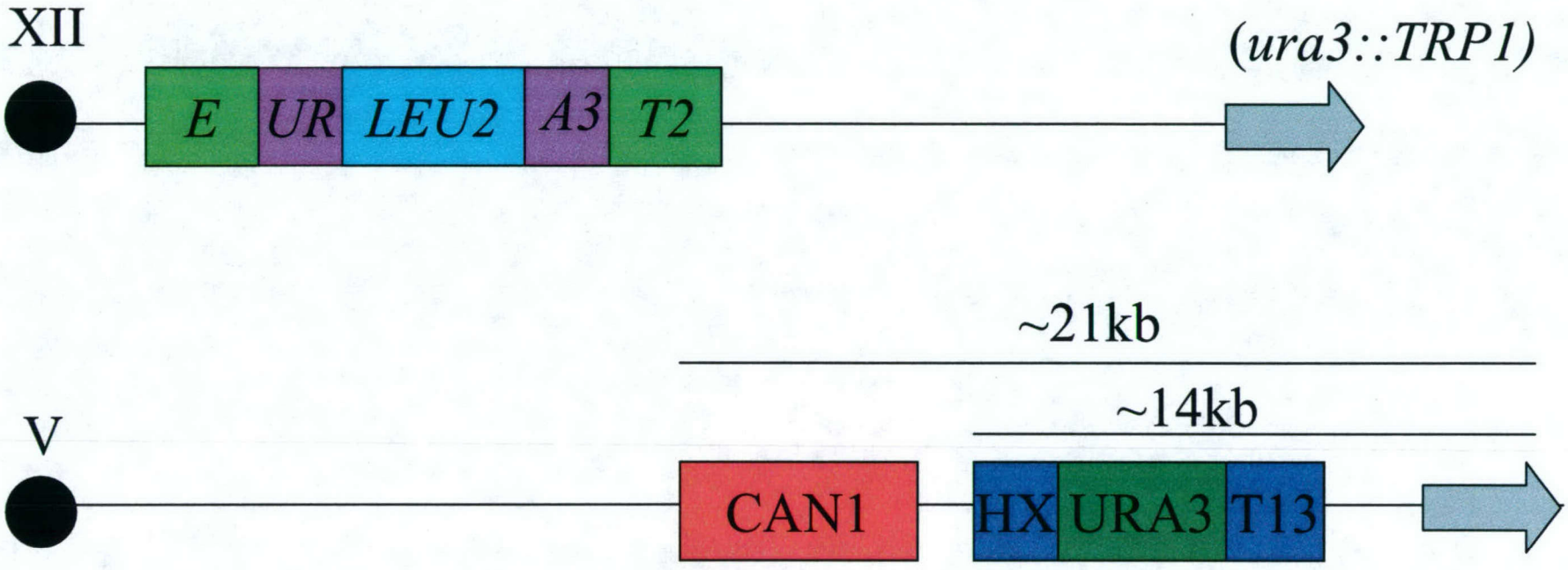


Fig. 5 Mutation & GCR assay



- Examined this strain for mutations at *CAN1* and GCR by plating to canavanine and *URA3* plates respectively as they grow over time.

Fig. 6 *CAN1* Mutations/generation

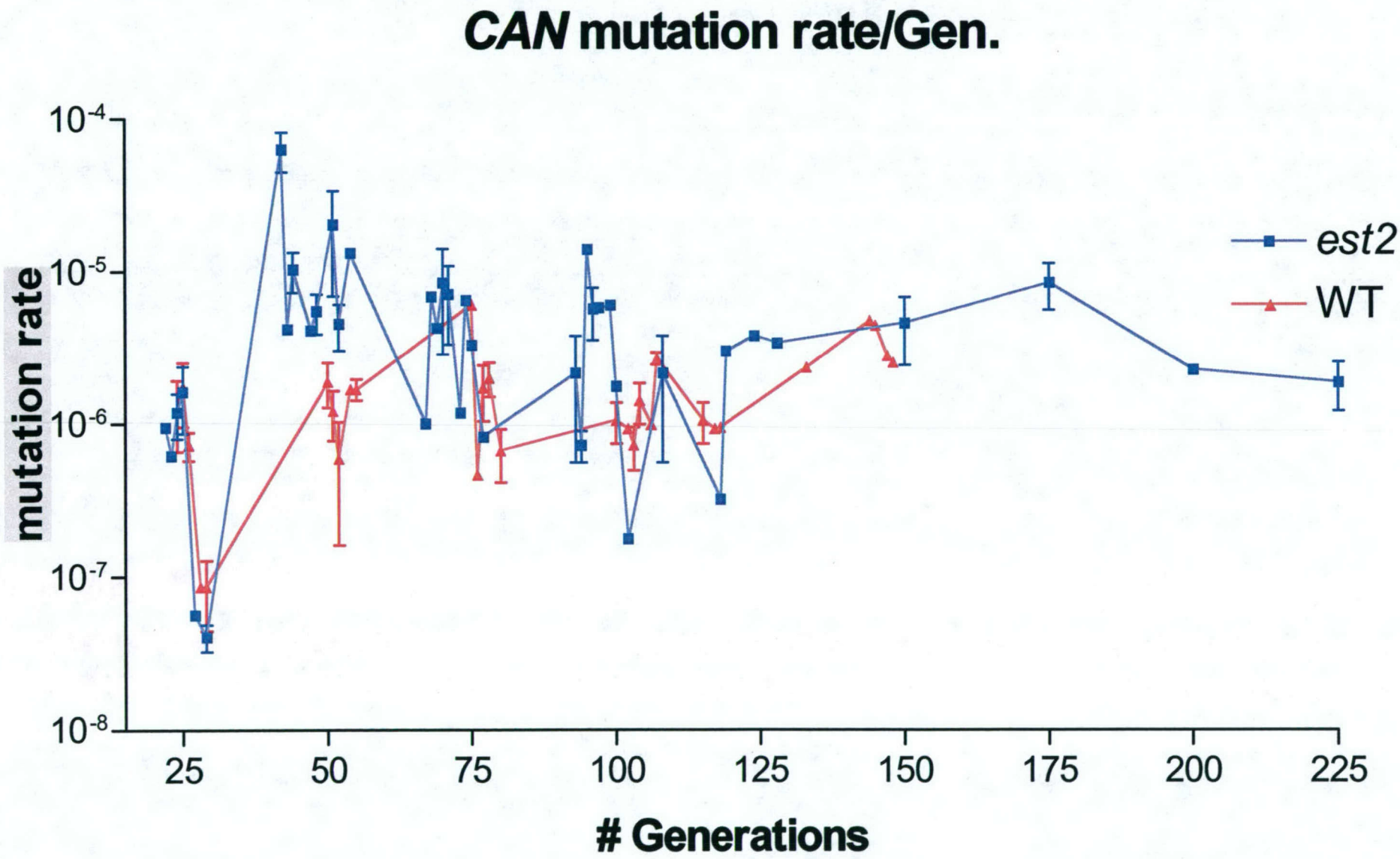


Fig. 7 GCR rate/generation